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(54) Title: PHARMACEUTICAL COMPOUNDS FOR THE INHIBITION OF HEPATITIS C VIRUS NS3 PROTEASE

(57) Abstract

Peptidic inhibitors of hepatitis C virus NS3 protease are disclosed which are based on the P and P' regions of the natural substrate. The P' part of the inhibitor is optimised to achieve maximum binding energy through interaction with the S' region of the enzyme. By selecting amino acids such that the inhibitor is substantially not cleavable by the NS3 protease inhibitors having potency in the low nanomolar to sub-nanomolar range can be achieved.

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PHARMACEUTICAL COMPOUNDS FOR THE INHIBITION OF HEPATITIS C VIRUS NS3 PROTEASE

Technical Field

This invention relates to compounds which can act as inhibitors of the hepatitis C virus (HCV) NS3 protease, to uses of such compounds and to their preparation.

Background Art

The hepatitis C virus (HCV) is the major causative agent of parenterally-transmitted and sporadic non-A, non-B hepatitis (NANB-H). Some 1% of the human population of the planet is believed to be affected. Infection by the virus can result in chronic hepatitis and cirrhosis of the liver, and may lead to hepatocellular carcinoma. Currently no vaccine nor established therapy exists, although partial success has been achieved in a minority of cases by treatment with recombinant interferon-α, either alone or in combination with ribavirin. There is therefore a pressing need for new and broadly-effective therapeutics.

Several virally-encoded enzymes are putative targets for therapeutic intervention, including a metalloprotease (NS2-3), a serine protease (NS3), a helicase (NS3), and an RNA-dependent RNA polymerase (NS5B). The NS3 protease

is located in the N-terminal domain of the NS3 protein, and is considered a prime drug target since it is responsible for an intramolecular cleavage at the NS3/4A site and for downstream intermolecular processing at the NS4A/4B, NS4B/5A and NS5A/5B junctions.

Previous research has identified classes of peptides, in particular hexapeptides, showing degrees of activity in inhibiting the NS3 protease. The aim of the present invention is to provide further compounds which exhibit similar, and if possible improved, activity.

According to the nomenclature of Schechter & Berger (1967, Biochem. Biophys. Res. Commun. 27, 157-162) cleavage sites in substrates for the NS3 protease are designated P6-P5-P4-P3-P2-P1...P1′-P2′-P3′-P4′-, with each P representing an amino acid, and the scissile bond lying between P1 and P1′. Corresponding binding sites on the enzyme are indicated as S6-S5-S4-S3-S2-S1...S1′-S2′-S3′-S4′.

The present applicant has previously disclosed so called product inhibitors which are based on the P-region of the natural cleavage sites and which have been optimised to low nanomolar potency ((1998) Biochemistry 37: 8899-8905 and (1998) Biochemistry 37: 8906-8914). These inhibitors extract much of their binding energy

from the C-terminal carboxylate, the remaining interactions with NS3 being similar to the ones used by the natural substrates, including binding in the S_1 pocket and the prominent electrostatic interaction of the P6-P5 acidic couple.

At variance with the P region, the P′ region of the substrate, while being important for catalysis, does not influence significantly ground-state binding to the enzyme as expressed by the Km value. In other words, binding energy released by the substrate interaction with the enzyme to form an initial non-covalent complex is essentially due to the interaction of the residues of the P region; the P′region residues contribute to a lesser extent to the binding energy. Accordingly, peptides based on the P′region of the natural substrates (spanning residues P_1'-P_{10}') do not inhibit NS3 to any significant extent. This notwithstanding, inspection of the crystal structure of NS3 with or without 4A (and more recently of the NMR structure of NS3) shows the presence of binding pockets in the S′ region which might be exploited for the binding of active-site directed inhibitors. S′-binding ligands would therefore display a range of interactions with the enzyme different from the ones used by the substrate, and represent a novel class of NS3 inhibitors.

Landro et al in (1997) Biochemistry 36, 9340-9348 synthesized certain non-cleavable decapeptides based on the NS5A/5B cleavage site by substituting the P_1' serine by a bulky cyclic aromatic (tetrahydroisoquinoline-3-carboxylic acid) or smaller cyclic alkyl compound (proline or pipecolinic acid). They then investigated the interaction of these decapeptides with the substrate binding site of NS3 either in the presence or absence of NS4A cofactor. By looking at the effect of truncation at either the P or P′ side of the molecule they concluded that most of the binding energy of the decapeptide is due to interactions with NS3-NS4A complex on the P side of the molecule. Truncation on the P′ side produced a relatively large effect in the presence of NS4A cofactor, but less when NS4A was absent. They concluded that the P4′ substrate Tyr residue present in their molecules was in close proximity, or in direct contact with NS4A and that this residue contributes significantly to binding in the presence of NS4A.

The present inventors have developed inhibitors which are more powerful than those described by Landro et al because they have better binding on their P′ side. In other words, the inhibitors take advantage of binding to the S′ region in addition to binding to the S-region of NS3. By varying the P′ amino acid residues, the present

inventors have shown that the binding energy which may be extracted from S′-region binding is substantial, since inhibitors with optimised and non-optimised P′-regions differ in potency > 1000-fold. Since no activity was present in any of the peptides corresponding to the isolated P′-region, optimisation of an S′-binding fragment was pursued in the context of non-cleavable decapeptides spanning P_6-P_4'.

The inventors found that, by replacing Landro′s P4′ Tyr residue by leucine the effectiveness of the decapeptides as NS3 protease inhibitors could be enhanced. Although it had been previously shown that leucine in position P4′ is better than tyrosine in a decapeptide substrate cleavable by NS3 (Urbani et al (1997) J. Biol. Chem <u>272</u>, 9204-9209), this is the first showing that the same applies to decapeptide inhibitors which are not cleaved under the influence of the enzyme. By optimising the P4′ residue and then the P2′-P3′ fragment and using these together with an optimised P region the inventors have arrived at oligopeptides which show potency in the low nanomolar-subnanomolar range.

Disclosure of the Invention

According to a first aspect of the present invention there is provided a compound having the formula (I)

(written from N-terminus to C-terminus):

$$\text{Pep-A}'\text{-B}'\text{-C}'\text{-D}' \qquad \text{(I)}$$

wherein "Pep" is a peptide or peptide analogue capable of binding to HCV NS3 protease; in particular, it is capable of binding in the S-region of the protease;

A′ is proline which is optionally substituted, for instance with one to three substituent groups;

B′ is an amino acid or amino acid analogue having a non polar side chain. Preferably, the side chain is an alkyl, aryl or aralkyl group containing 3 to 10, particularly 4 to 8 carbon atoms;

C′ is an amino acid or amino acid analogue having a polar side group. Examples of polar side group may contain between 2 and 10, preferably 2 to 6 carbon atoms;

D′ is leucine, or less preferably another amino acid with a non-polar aliphatic side chain, such as valine, isoleucine, norleucine or methionine. Alternatively, it is a short peptide or peptide analogue having one of these amino acids, especially leucine at its N terminus. The short peptide or peptide analogue may, for instance

comprise 2 to 6, preferably 2 to 4 amino acids or amino acid analogues.

As used herein, the term "amino acid analogue" includes organic compounds containing an amino and a carboxylic acid group, for instance arranged α- to each other, and which do not necessarily occur in nature.

The Pep-A bond of the compound of formula (I) is substantially uncleavable by HCV NS3 protease. For instance, it is preferable that no cleavage be detectable using the assay described below under the heading "Substrate Assay".

Pharmaceutically acceptable salts of the compound of formula (I), as well as derivatives, such as esters are within the scope of the present invention.

Preferably, the compound of formula (I) is N-terminally acylated, especially acetylated, although other derivatives of the N-terminus are also possible, for instance N-terminal sulphoxide, sulphonamide, urethane or urea derivatives.

Preferably, the compound of formula (I) is C-terminally amidated. However, the C-terminus may be an

underivatised carboxylic acid group. Alternatively, other C-terminal groups may be present.

Assuming no substitution of the proline residue at A′ is present, then a preferred C-terminal portion of the compound of formula I is:

Pro-B′-C′-Leu

possibly with a short C terminal extension at Leu.

Preferred examples of the amino acid, or analogue, B′ for inclusion in compounds of the first aspect of the invention, include:

β-cyclohexylalanine, phenylglycine, homophenylalanine and norleucine; other possibilities, though less preferred, are leucine, methionine, norvaline, and β-cyclopropylalanine. Of all these, cyclohexylalanine and phenyl glycine are most preferred.

Examples of the amino acid or analogue, C′ include aspartic acid, glutamic acid, γ-carboxyglutamic acid, glutamine, asparagine, and hydroxyproline. Slightly less preferred are N-β-Aloc-diaminobutyric acid, thiazolylalanine, methionine sulfoxide, pyridylalanine

and serine. Of all of these aspartic acid is most preferred.

The following combinations of amino acid residues at B′ and C′ are preferred, of which the combination of cyclohexylalanine and aspartic acid is especially preferred.

TABLE 1

B′	C′
Cha	Ser
Cha	Asp
Nle	Asp
Hof	Asp
Phg	Asp
Cha	Gln
Nle	Gln
Hof	Gln
Cha	Hyp
Nle	Hyp
Hof	Hyp
Nle	Ser

Notes: Cha = β-cyclohexylalanine.
Nle = norleucine.
Phg = phenylglycine.
Hof = homophenylalanine.
Hyp = hydroxyproline.

When the residue D′ is leucine (or other amino acid)

with a small peptide as C-terminal extension the peptide may be chosen by comparison with the corresponding P′ portion of natural substrates.

The residues A′,B′,C′ and D′ may have D- or L-stereochemistry, although L-stereochemistry is, in general, preferred for each.

As regards the Pep part of the compound of formula (I) this is particularly preferably a peptide or peptide analogue capable of binding to HCV NS3 protease, even in the absence of the C-terminal residues A′-B′-C′-D′, for instance when Pep carries just a carboxylic acid group at the C terminus. For example, when tested in the inhibition assay described below the fragment Pep-OH preferably has an IC_{50} below 100μM, e.g. below 20μM, particularly below 10μM and, optimally, of less than 1μM. Preferably, Pep is a hexa-, penta- or tetra peptide having formula II below:

F-E-D-C-B-A (II)

wherein: A is an amino acid or amino acid analogue having a relatively small (C_1-C_6) aliphatic side chain. Possible choices for this group include cysteine, aminobutyric acid (Abu) (including di- and tri-fluoro

Abu), norvaline, allylglycine and alanine, any of which may be N-methylated. Of these, cysteine and the fluorinated aminobutyric acids are preferred choices for A.

B is an amino acid or analogue having a non-polar or acidic side chain. Some amino acids having polar but uncharged side groups may also be suitable. Examples of suitable amino acids include glutamic and aspartic acid, glycine and methyl glycine, 2-amino butyric acid, alanine, isoleucine, valine, leucine, cysteine, naphthylalanine and β-cyclohexylalanine. Of these, cyclohexylalanine is particularly preferred.

C is an amino acid or amino acid analogue having a non-polar or acidic side chain. For instance, the examples of such amino acids given above for B apply also to C. In this case isoleucine and glutamic acid are particularly preferred.

D is usually an amino acid or amino acid analogue having a hydrophobic side group such as methionine, isoleucine, leucine, norleucine, valine, methyl valine, phenylglycine or, diphenylalanine. Among these leucine and, particularly, diphenylalanine are preferred. Some polar amino acids which include hydrophobic portions,

such as tyrosine, thienylalanine, and chlorophenylalanine may be suitable.

E together with F may be absent, but if present is generally an amino acid or amino acid analogue having an acidic side chain. Preferred examples are glutamic and aspartic acid, with the former being preferred. E may, alternatively, be an amino acid or analogue having a non-polar, or polar but uncharged side chain. Of the non-polar amino acids, phenylalanine, diphenylalanine, isoleucine and valine are preferred, especially the D-enantiomers. Among the polar amino acids suitable examples are tyrosine and 4-nitrophenylalanine. Alternatively, where F is absent (see below), E may be a dicarboxylic acid containing up to 6 carbon atoms and lacking the amino group of acidic amino acids. Suitable examples are glutaric and succinic acid.

F may be absent (either by itself, or together with E), but when present is an amino acid or analogue having an acidic side chain. Aspartic acid is preferred, although glutamic acid is another possibility. Like E, F may also be a dicarboxylic acid containing up to 6 carbon atoms, and lacking the amino group of acidic amino acids. Examples are glutaric and succinic acid.

Of residues E and F preferably at least E is present. Particularly preferably both are present.

The amino acids and analogues A-F may be either L- or D- enantiomers though L- is generally preferred for all residues. In some cases it may be beneficial for one or other of the residues to be D- while the rest are L-. In particular it may be advantageous for E to be D-glu.

Preferred examples of the peptide "Pep" are listed below in Tables 2 and 3 together with their IC_{50} values when unextended at the C-terminus. Except for the compounds having a succinyl residue at the N-terminus, all compounds tested were N-acetylated at the N-terminus.

TABLE 2

Exp No.	Sequence	IC_{50} (μM)
1	Asp Glu Met Glu Glu Cys	1.0
2	Asp Glu Met Glu Glu D-Cys	4.0
3	Asp Glu Met Glu Glu Abu	5.8
4	Met Glu Glu Cys	150.0
5	Glu Met Glu Glu Cys	21.0
6	Glu Asp Val Val Cys Cys	5.3
7	Glu Asp Val Val Abu Cys	2.8
8	Asp Glu Val Val Cys Cys	2.1
9	Glu Asp Val Val Gly Cys	20.0
10	Asp Glu Met Glu Glu Alg	12.0
11	Glu Asp Val Val MGly Cys	21.0
12	Glu Asp MVal Val Abu Cys	1.3
13	GluS Met Glu Glu Cys	1.3
14	AsGlu Met Glu Glu Cys	0.6
15	Asp Glu Met Glu Leu Cys	1.1
16	Asp Glu Met Glu Cha Cys	0.3
17	Asp Glu Met Glu Nap Cys	0.8
18	AspS Val Val Abu Cys	4.6
19	Asp Glu Met Glu Glu Cys(Me)	16.7
20	Asp Glu Val Glu Cha Cys	0.33

21	Asp Glu Ile Glu Cha Cys	0.12
22	Asp Glu Tyr Glu Cha Cys	0.24
23	Asp Glu Phe Glu Cha Cys	0.42
24	Asp Glu Leu Glu Cha Cys	0.12
25	Asp Glu Cha Glu Cha Cys	0.14
26	Asp Glu Nle Glu Cha Cys	0.22
27	Asp Glu Tha Glu Cha Cys	0.87
28	Asp Glu FCI Glu Cha Cys	0.3
29	Asp Glu Phg Glu Cha Cys	0.12
30	Asp Glu Dif Glu Cha D-Cys	3.4
31	Glu Dif Glu Cha Cys	1.4
32	Dif Glu Cha Cys	30.0
33	Asp MGlu Leu Glu Cha Cys	1.0
34	Asp Glu Dif Glu Cha DHAla	7.1
35	Asp Glu Met Glu Glu Cpc	9.0
36	Glu Dif Ile Cha Cys	2.5
37	Dif Ile Cha Cys	100.0
38	Asp Glu Met Glu Glu CnAla	19.0
39	Asp Glu Leu Glu Cha Abu	1.6

40	Asp Glu Leu Glu Cha Val	4.0
41	Asp Glu Leu Glu Cha Nva	1.3
42	Asp-Asp-Leu-Glu-Cha-Cys	0.290
43	Asp-Fno-Leu-Glu-Cha-Cys	0.240
44	Asp-Tyr-Leu-Glu-Cha-Cys	0.135
45	Asp-(D)Phe-Leu-Glu-Cha-Cys	0.820
46	Asp-(D)Tyr-Leu-Glu-Cha-Cys	0.680
47	Asp-(D)Val-Leu-Glu-Cha-Cys	0.470
48	Asp-(D)Ile-Leu-Glu-Cha-Cys	0.330
49	Asp-(D)Dif-Leu-Glu-Cha-Cys	0,276
50	Asp-(D)Asp-Leu-Glu-Cha-Cys	0,122
51	Asp-Glu-Dap(N-b-Dns)-Glu-Cha-Cys	0,4

Particularly preferred examples of Pep, together with their IC_{50}s (in µM) are set out below in Table 3 are:

TABLE 3

Most preferred:

1	**Asp Glu Dif Glu Cha Cys**	0.05
2	Asp Glu Leu Val Cha Cys	0.08
3	Asp Glu Leu Ile Cha Cys	0.06
4	Asp Glu Dif Ile Cha Cys	0.06
5	Asp-Gla-Leu-Glu-Cha-Cys	0.055
6	Asp-(D)Glu-Leu-Glu-Cha-Cys	0.045
7	Asp-(D)Gla-Leu-Ile-Cha-Cys	0.0015
8	Glu-Leu-Glu-Cha-Cys	1.3
9	(D)Glu-Leu-Glu-Cha-Cys-(Pro-Cha-Asp-Leu)	0.080*
10	Succinyl Glu-Leu-Ile-Cha-Cys	
11	Succinyl (D)Glu-Leu-Glu-Cha-Cys-(Pro-Cha-Asp-Leu)	0.0040*
12	Asp-(D)Glu-Leu-Ile-Cha-Cys	
13	Asp-(D)Glu-Leu-Ile-Cha-Cys-(Pro-Cha-Asp-Leu)	<0.0002*

* Tested only as decapeptides

In these compounds:

Alg	=	allylglycine.
MGly	=	methylglycine.
MVal	=	methylvaline.
Abu	=	2-aminobutyric acid.
GluS	=	N-succinylglutamic acid.
AsGlu	=	Glutamic acid having N-terminal acylsulphonamide.
Cha	=	β-cyclohexylalanine.
Nap	=	naphthylalanine.
AspS	=	N-succinylaspartic acid.
Nle	=	norleucine.
Dif	=	3,3-diphenylalanine.
Tha	=	2-thienylalanine.
FCI	=	4-chlorophenylalanine.
Phg	=	phenylglycine.
CysMe	=	S-methylcysteine.

Cys(ACS)	=	Cysteine with C-terminal acylsulphonamide.
DHAla	=	dehydroalanine.
Cpc	=	1-amino-1-cyclopentane carboxylic acid.
CnAla	=	cyanoalanine.
MGlu	=	N-methylglutamic acid.
Fno	=	4-nitrophenylalanine.
Gla	=	γ-carboxyglutamic acid.
Dap	=	β-diaminopropionic acid.
Dns	=	dansyl(5-dimethylamino-1-naphthalene-sulfonyl).

Examples of compounds of the present invention may be effective as inhibitors of NS3 protease at micromolar or nanomolar levels. Preferably, the IC_{50}, as measured in the assay described below is less than 100nM, particularly preferably less than 20nM and, optimally, less than 5nM.

According to a second aspect, the present invention provides a compound, salt or derivative according to the first aspect, for use in any therapeutic method, preferably for use in inhibiting the HCV NS3 protease, and/or for use in treating or preventing hepatitis C or a related condition. By "related condition" is meant a condition which is or can be caused, directly or indirectly, by the hepatitis C virus, or with which the HCV is in any way associated.

According to a third aspect the present invention provides the use of a compound or derivative according to the first aspect in the manufacture of a medicament for the treatment or prevention of hepatitis C or a related condition.

A fourth aspect of the invention provides a pharmaceutical composition which includes one or more compounds or derivatives according to the first aspect.

The composition may also include pharmaceutically acceptable adjuvants such as carriers, buffers, stabilisers and other excipients. It may additionally include other therapeutically active agents, in particular those of use in treating or preventing hepatitis C or related conditions.

The pharmaceutical composition may be in any suitable form, depending on the intended method of administration. It may for example be in the form of a tablet, capsule or liquid for oral administration, or of a solution or suspension for administration parenterally.

According to a fifth aspect of the invention, there is provided a method of inhibiting HCV NS3 protease activity, and/or of treating or preventing hepatitis C or a related condition, the method involving administering to a human or animal (preferably mammalian) subject suffering from the condition a therapeutically or

prophylactically effective amount of a composition according to the fourth aspect of the invention, or of a compound or derivative according to the first aspect. "Effective amount" means an amount sufficient to cause a benefit to the subject or at least to cause a change in the subject's condition.

The dosage rate at which the compound, derivative or composition is administered will depend on the nature of the subject, the nature and severity of the condition, the administration method used, etc. Appropriate values can be selected by the trained medical practitioner. Preferred daily doses of the compounds are likely to be of the order of about 1 to 100 mg. The compound, derivative or composition may be administered alone or in combination with other treatments, either simultaneously or sequentially. It may be administered by any suitable route, including orally, intravenously, cutaneously, subcutaneously, etc. Intravenous administration is preferred. It may be administered directly to a suitable

site or in a manner in which it targets a particular site, such as a certain type of cell - suitable targeting methods are already known.

A sixth aspect of the invention provides a method of preparation of a pharmaceutical composition, involving admixing one or more compounds or derivatives according to the first aspect of the invention with one or more pharmaceutically acceptable adjuvants, and/or with one or more other therapeutically or prophylactically active agents.

According to a seventh aspect of the invention there is provided a method of producing the compounds of formula I. These compounds may be generated wholly or partly by chemical synthesis beginning from individual, preferably protected, amino acids or oligopeptides and using known peptide synthesis methods.

Modes for Carrying Out the Invention

Embodiments of the invention are exemplified below by way of illustration only.

EXAMPLES

(1) Synthesis

The synthesis of one of the compounds of the present invention is described below. Other compounds may be synthesized by an analogous method.

Synthesis of Ac-Asp-(D)Glu-Leu-Ile-Cha-Cys-Pro-Cha-Asp-Leu-Pro-Tyr-Lys(N^{ϵ}-Ac)-NH_2

The synthesis was performed on solid phase by the continuous-flow Fmoc-polyamide method (Atherton, E. and Sheppard, R. C. (1989) Solid phase peptide synthesis. A practical approach, IRL Press, Oxford.). The resin used was Tentagel™ derivatised with a modified Rink amide

linker p-[(R,S)-α-[1-(9H-Fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetic acid (Rink, H. (1987) *Tetrahedron Lett.* **28**, 3787-3789; Bernatowicz, M. S., Daniels, S. B. and Koster, H. (1989) *Tetrahedron Lett.* **30**, 4645-4667). All the coupling reactions were performed for 30 min with 5-fold excess of activated amino acid over the resin free amino groups, using Fmoc-amino acid/PyBOP/HOBt/DIEA (1:1:1:2) activation; double coupling was used for the cysteine residue. At the end of the assembly, the dry peptide-resin was treated with trifluoroacetic acid/water/triisopropylsilane (92.5:5:2.5) for 1.5h at room temperature; the resin was filtered out and the peptide precipitated with cold methyl t-Bu ether; the precipitate was redissolved in 50% water/acetonitrile containing 0.1%TFA and lyophillised.

Purification to >98% homogeneity was achieved through preparative HPLC on a Waters RCM (C-18) column (100 X 25 mm, 15mm) using as eluents (A) 0.1% trifluoroacetic acid in water and (B) 0.1%

trifluoroacetic acid in acetonitrile. The gradient used was 40%B isocratic for 5 min, then 40-60%B over 20 min, flow rate 30 ml/min; the fractions were analysed by HPLC (column: Beckman Ultrasphere, C-18, 25 X 4.6 mm, 5mm; gradient: 35-65%B in 20 min, same eluents as the preparative run, flow 1ml/min) and those containing the pure material were pooled and lyophilised (yield=50%). The Mass spectrum was acquired on a Perkin-Elmer API-100 spectrometer: MS= 1695.03 (calc.) 1694.6 (found).

(2) Inhibition Assay

The ability of the compounds to inhibit NS3 protease was evaluated using an NS3/4A complex comprising the NS3 protease domain and a modified form of the NS4A peptide, Pep 4AK [KKKGSVVIVGRIILSGR(NH_2)]. As substrate, a substrate peptide 4AB [DEMEECASHLPYK] based on the sequence of the NS4A/NS4B cleavage site of the HCV polyprotein, was used.

Cleavage assays were performed in 57µl 50 mM Hepes pH7.5, 1 % CHAPS, 15 % glycerol, 10 mM DTT (buffer A), to which 3µl substrate peptide were added. As protease co-factor a peptide spanning the central hydrophobic core (residues 21-34) of the NS4A protein, Pep4AK [KKKGSVVIVGRIILSGR(NH_2)] was used. Buffer solutions containing 80 µM Pep4AK were preincubated for 10 minutes with 10-200 nM protease and reactions were started by addition of substrate. Six duplicate data points at different substrate concentrations were used to calculate kinetic parameters. Incubation times were chosen in order to obtain <7% substrate conversion and reactions were stopped by addition of 40 µl 1 % TFA. Cleavage of peptide substrates was determined by HPLC using a Merck-Hitachi chromatograph equipped with an autosampler. 80 µl samples were injected on a Lichrospher C18 reversed phase cartridge column (4 x 74mm, 5µm, Merck) and fragments were separated using a 10-40 % acetonitrile gradient a 5%/min using a flow rate of 2.5ml/min. Peak detection was accomplished by monitoring both the

absorbance at 220nm and tyrosine fluorescence (λ_{ex} = 260 nm, λ_{em} = 305nm). Cleavage products were quantitated by integration of chromatograms with respect to appropriate standards. Kinetic parameters were calculated from nonlinear least-squares fit of initial rates as a function of substrate concentration with the help of a Kaleidagraph software, assuming Michaelis-Menten kinetics.

K_1 values of peptide inhibitors were calculated from substrate titration experiments performed in the presence of increasing amounts of inhibitor. Experimental data sets were simultaneously fitted to eq.1 using a multicurve fit macro with the help of a Sigmaplot software:

$$V = (V_{max}S)/(K_m(1+K_1/I)+S); \qquad \text{(eq.1)}$$

Alternatively, K_i values were derived from IC50

values, calculated using a four-parameter logistic function, according to eq.2:

$$IC50 - (1+S/K_m)K_i \qquad \text{(eq.2)}$$

The table below sets out the IC_{50} values for a variety of peptides tested in this assay and establishes that several optimised compounds of the present invention are active at nanomolar or subnanomolar levels. All the compounds tested - except for compound 26 which has a succinyl residue at the N-terminus- were tested as their N-acetyl derivatives.

Some of these compounds are the most potent in vitro inhibitors of HCV protease described to date. They are reversible, non covalent inhibitors which do not contain an electrophilic ("serine-trap") moiety in the molecule. They bind to both the S and S′ region of the enzyme, and this makes them suitable for developing competition

binding assays, since they would be competitive with compounds binding to either the S or the S′ region of the enzyme.

Table 4

Ex. No	Sequence	IC_{50} (nM)
1	Glu Asp Val Val Abu Cys Pro Nle Ser Tyr	8500
2	Glu Asp Val Val Abu Cys (Me)Ala Nle Ser Tyr	3500
3	Asp Glu Dif Ile Cha Abu Ala Ser His Leu	29000
4	Asp Glu Dif Ile Cha Abu (Me)Ala Ser His Leu	29000
5	Asp Glu Dif Ile Cha (Me)Abu Ala Ser His Leu	8000
6	Asp Glu Dif Ile Cha (Me)Abu (Me)Ala Ser His Leu	3800
7	Asp (D)Glu Dif Ile Cha (Me)Abu (Me)Ala Ser His Leu	3100
8	Asp (D)Glu Leu Ile Cha Abu (Me)Ala Ser His Leu	5000
9	Asp Glu Dif Ile Cha Cys Pro Nle Ser Tyr	876
10	Glu Dif Ile Cha Cys Pro Nle Ser Leu	64
11	Asp Glu Dif Ile Cha Cys Pro Cha Ser Leu	23
12	Asp Glu Dif Ile Cha Cys Pro Cha Asp Leu	1.3
13	Asp Glu Dif Ile Cha Cys Pro Phg Asp Leu	7
14	Asp Glu Dif Ile Cha Cys Pro Nle Asp Leu	1.8
15	Asp Glu Dif Ile Cha Cys Pro Hof Asp Leu	1.8
16	Asp Glu Dif Ile Cha Cys Pro Cha Gln Leu	14
17	Asp Glu Dif Ile Cha Cys Pro Nle Gln Leu	32
18	Asp Glu Dif Ile Cha Cys Pro Hof Gln Leu	18

19	Asp Glu Dif Ile Cha Cys Pro Cha Hyp Leu	11
20	Asp Glu Dif Ile Cha Cys Pro Nle Hyp Leu	26
21	Asp Glu Dif Ile Cha Cys Pro Hof Hyp Leu	15
22	Asp (D)Glu Leu Ile Cha Cys Pro Nle Ser Leu	10
23	Asp Glu Dif Ile Cha Cys Pro Cha Asp Leu PYK(Ac)	0.85
24	Asp (D)Glu Leu Ile Cha Cys Pro Cha Asp Leu PYK(Ac)	< 0.2
25	Asp (D)Glu Leu Ile Cha Cys Pro Cha Asp Leu	< 0.2
26	Suc-(D)Glu Leu Ile Cha Cys Pro Cha Asp Leu	4
27	Asp (D)Glu Leu Glu Cha Cys Pro Cha Asp Leu	0.63
28	(D)Glu Leu Glu Cha Cys Pro Cha Asp Leu	80
29	Asp (D)Glu Leu Glu Cha Ala Pro Cha Asp Leu	17
30	Asp (D)Glu Leu Ile Cha Cys Pro Nle Ser Leu	10

Abbreviations used in Table I:

Abu = aminobutyric acid

Cha = β-cyclohexylalanine

Hof = homophenylalanine

Hyp = hydroxyproline

Lys(Ac) or K(Ac) = Nε-Acetyl-Lysine

Nle = norleucine

Phg = phenylglycine

Sta = statine [(3S,4S)-4-amino-3-hydroxy-6-methylheptanoic acid]

Dif = 3,3-diphenylalanine

Suc=succinyl

N-methylation is indicated as (Me) preceding the three-letter code of the amino acid

PYK = proline-tyrosine-lysine

(3) Substrate Assay

In order to determine whether or not an inhibitor molecule was a substrate for HCV NS3 protease a modified

version of the cleavage assay described above was employed using, as before, an NS3/4A complex comprising the NS3 protease domain and a modified form of the NS4A peptide, Pep4AK [KKKGSVVIVGRIILSGR(NH_2)].

1µM of the enzyme complex was incubated for 16hrs in the presence of 10µM inhibitor as a candidate substrate peptide. Assays were performed in 57µl 50 mM Hepes pH7.5, 1% CHAPS, 15% glycerol, 10 mM DTT.

After this time HPLC was used to separate any peptides resulting from cleavage and separated cleavage products detected.

Samples were analysed by HPLC on a Beckman 0.46 x 25 cm C18 reversed phase column equilibrated in 95% solvent A (0.1% TFA in H20) and 5% solvent B (0.1% TFA in acetonitrile) at a flow rate of 1 ml/min. Samples were eluted from this column with a linear gradient from 5% to

90% of B in 45 minutes. Peak detection was accomplished by monitoring absorbance at 220 nm.

CLAIMS:

1. A compound of formula (I), or a pharmaceutically acceptable salt or derivative thereof:

$$\text{Pep-A'-B'-C'-D'} \qquad (I)$$

wherein formula (I) is written from N-terminus on the left to C-terminus at the right and:

"Pep" is a peptide or peptide analogue capable of binding to HCV NS3 protease;

A' is a proline residue which is optionally substituted;

B' is an amino acid or amino acid analogue having a non-polar side chain;

C' is an amino acid or amino acid analogue having a polar side chain; and

D' is selected from leucine, other amino acids or amino acid analogues having a non-polar aliphatic side chain, and peptides of 2 to 6 amino acids having leucine or other amino acid or amino acid analogue with a non-polar aliphatic side chain as N-terminal residue;

and wherein the bond between Pep and A' is substantially uncleavable by HCV NS3 protease.

2. The compound of claim 1, a pharmaceutically acceptable salt or derivative thereof which is C-

terminally amidated.

3. The compound of claim 1 or claim 2, a pharmaceutically acceptable salt or derivative thereof which is N-terminally acylated.

4. A compound, salt or derivative according to any one of claims 1 to 3 wherein A'-B'-C'-D' is a tetrapeptide of formula:

Pro-B'-C'-Leu

wherein B' and C' are as defined in claim 1.

5. A compound, salt or derivative according to any one of the previous claims wherein B' is selected from: β-cyclohexylalanine, phenylglycine, homophenylalanine, norleucine, leucine, methionine, norvaline and β-cyclopropylalanine.

6. A compound, salt or derivative according to claim 5 wherein B' is selected from cyclohexylalanine and phenylglycine.

7. A compound, salt or derivative according to any one of the previous claims wherein C' is selected from: aspartic acid, glutamic acid, γ-carboxyglutamic acid,

glutamine, asparagine, hydroxyproline, N-β-Aloc-diaminobutyric acid, thiazolylalanine, methionine sulfoxide, pyridylalanine and serine.

8. A compound, salt or derivative according to claim 7 wherein C' is aspartic acid.

9. A compound, salt or derivative according to any one of the preceding claims, wherein the combination of amino acids B'C' is selected from:

Cha-Ser

Cha-Asp

Nle-Asp

Hof-Asp

Phg-Asp

Cha-Gln

Nle-Gln

Hof-Gln

Cha-Hyp

Nle-Hyp

Hof-Hyp

Nle-Ser

10. A compound, salt, or derivative according to any one of the preceding claims wherein Pep-OH is capable of

binding HCV NS3 protease, in the absence of the C-terminal residues A'-B'-C'-D', and has an IC_{50} below 100μM in an inhibition assay.

11. A compound, salt, or derivative according to any one of the preceding claims wherein Pep is a hexa-, penta- or tetra-peptide having formula (II) below:

F-E-D-C-B-A

wherein: A is an amino acid or amino acid analogue having an aliphatic side chain of form 1 to 6 carbon atoms;

B is an amino acid or analogue having a non-polar, acidic, or polar but uncharged side group;

C is an amino acid or amino acid analogue having a non-polar or acidic side chain;

D is an amino acid or amino acid analogue having a hydrophobic side group;

E together with F may be absent, but if present is an amino acid or amino acid analogue having an acidic side chain, non-polar side chain or polar, but uncharged side chain, or is a dicarboxylic acid containing up to 6 carbon atoms and lacking the amino group of acidic amino acids;

and F may be absent (either by itself, or together with E) but when present is an amino acid or analogue

having an acidic side chain or is a dicarboxylic acid containing up to 6 carbon atoms.

12. A compound according to claim 11 wherein:

A is selected from: cysteine, aminobutyric acid, di- and tri-fluoro aminobutyric acid, norvaline, allylglycine and alanine;

B is selected from: glutamic acid, aspartic acid, glycine, methyl glycine, 2-amino butyric acid, alanine, isoleucine, valine, leucine, cysteine, naphthylalanine and β-cyclohexylalanine;

C is selected from: glutamic acid, aspartic acid, glycine, methyl glycine, 2-amino butyric acid, alanine, isoleucine, valine, leucine, cysteine, naphthylalanine and β-cyclohexylalanine;

D is selected from: methionine, isoleucine, leucine, norleucine, valine, methylvaline, phenylglycine, diphenylalanine, tyrosine, thienylalanine, and chlorophenylalanine;

E is selected from: glutamic acid, aspartic acid, phenylalanine, diphenylalanine, isoleucine, valine, tyrosine, 4-nitrophenylalanine, glutaric acid and succinic acid;

and F is selected from: aspartic acid, glutamic acid, glutaric acid and succinic acid.

13. A compound, salt, or derivative, according to any one of the preceding claims for use in therapy.

14. A pharmaceutical composition comprising a compound, salt or derivative according to any one of the preceding claims and a pharmaceutically acceptable excipient, diluent or carrier.

15. Use of a compound, salt or derivative according to any one of the preceding claims in the manufacture of a medicament for the treatment or prevention of hepatitis C or a related condition.

16. A method of inhibiting HCV NS3 protease activity, and/or of treating or preventing hepatitis C or a related condition, comprising administering to a human or mammalian subject suffering from the condition a therapeutically or prophylactically effective amount of a composition according to claim 14, or of a compound of any one of claims 1 to 12.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/09207

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/18 A61K39/29 A61P31/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	P. INGALLINELLA ET AL.: "Potent Peptide Inhibitors of Human Hepatitis C Virus NS3 Protease Are Obtained by Optimizing the Cleavage Products" BIOCHEMISTRY, vol. 37, no. 25, 23 May 1998 (1998-05-23), pages 8906-8914, XP002134585 EASTON, PA US page 8911, left-hand column, paragraph 1 -page 8913, right-hand column, paragraph 3; table 1 ---- -/--	1,14-16

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 March 2000	12/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fuhr, C

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	A. URBANI ET AL.: "Substrate Specificity of the Hepatitis C Virus Serine Protease NS3" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 14, 4 April 1997 (1997-04-04), pages 9204-9209, XP002134586 BALTIMORE, MD US	1,14-16
A	J.A. LANDRO ET AL.: "Mechanistic Role of an NS4A Peptide Cofactor with the Truncated NS3 Protease of Hepatitis C Virus: Elucidation of the NS4A Stimulatory Effect via Kinetic Analysis and Inhibitor Mapping" BIOCHEMISTRY, vol. 36, no. 11, 5 August 1997 (1997-08-05), pages 9340-9348, XP002134587 EASTON, PA US page 9345, right-hand column, paragraph 3 -page 9347, right-hand column, paragraph 1; tables 3,4	1,14-16
A	C. STEINKÜHLER ET AL.: "Product Inhibition of the Hepatitis C Virus NS3 Protease" BIOCHEMISTRY, vol. 37, no. 25, 23 June 1998 (1998-06-23), pages 8899-8905, XP002134588 EASTON, PA US page 8903, left-hand column, last paragraph -page 8904, right-hand column, last paragraph; table 1	1,14-16

1

INTERNATIONAL SEARCH REPORT	International application No. PCT/EP 99/09207

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 16 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.